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The HBx oncoprotein of hepatitis B virus engages nucleophosmin to promote rDNA transcription and cellular proliferation

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ABSTRACT

The pleiotropic HBx oncoprotein of hepatitis B virus is well known to promote the expression of ribosomal RNAs and several host proteins that are known to support the development and progression of hepatocellular carcinoma (HCC). While overexpression of the nucleolar phosphoprotein, nucleophosmin (NPM), correlates with HCC progression, its upregulation by viral HBx and the resulting impact on perturbed nucleolar functions remain enigmatic. The present study shows that HBx up-regulates NPM levels and hijacks its functions to promote cellular proliferation. We found that HBx expression stabilizes NPM through post-translational modifications. Enhanced CDK2-mediated phosphorylation of NPM at Thr^{199} upon HBx expression prevented its proteolytic cleavage and provided resistance to apoptosis. Further, HBx directly interacted with the C-terminal domain of NPM and got translocated into the nucleolus where it facilitated the recruitment of RNA polymerase I transcriptional machinery onto the rDNA promoter. Our results indicate that HBx enhances rDNA transcription *via* a novel regulatory mechanism involving acetylation of NPM and the subsequent depletion of histones from the rDNA promoter. Enhanced production of ribosomal RNA resulting from co-expression of HBx and NPM promoted ribosome biogenesis, cellular proliferation and transformation. Taken together, our study strongly suggests an important role of NPM in mediating the oncogenic effects of HBx and the corresponding nucleolar perturbations induced by this viral oncoprotein.

1. Introduction

In eukaryotes, ribosome biogenesis is an extraordinarily orchestrated multistep process requiring the coordinated action of all three RNA polymerases and involving the assembly of ribosomal RNAs (rRNAs) and ribosomal proteins (RPs) [2,54]. After their synthesis in the cytoplasm, RPs are translocated into the nucleolus where along with rRNAs they are assembled into two ribosomal subunits. The rRNA synthesis and nucleolar import of RPs involve a complex interplay of several factors including the multifunctional nucleolar phosphoprotein, nucleophosmin (NPM). NPM is a ubiquitously expressed nucleolar protein that shuttles across different cellular compartments primarily due to the presence of nuclear localization, nucleolar localization and nuclear export signals [15]. It affects diverse cellular processes such as DNA replication and repair, transcription, chromatin remodeling, apoptosis, formation of mitotic spindle and centrosome duplication [37]. NPM also plays a lead role in ribosome biogenesis by engaging c-Myc to stimulate RNA Pol I-dependent transcription [35] and by exporting ribosomal proteins and mature ribosomes from nucleolus to the cytoplasm [4,39,60]. Besides, NPM may undergo several post-translational modifications such as phosphorylation, acetylation, and SUMOylation which affect its functions [38,45,51]. For example, the de-regulation of NPM phosphorylation may result in the formation of super-numerary centrosomes and missegregation of chromosomes leading to aneuploidy [12,28,44]. These changes are considered as hallmarks of many cancers [18].

Enhanced expression of NPM is associated with uncontrolled cellular proliferation and neoplastic transformation [9,15,16]. Most of the human cancers including hepatocellular carcinoma (HCC) are characterized by nucleolar hypertrophy and increased ribosome biogenesis [56]. The HBx oncoprotein of hepatitis B virus (HBV) which is implicated in the development of HCC, is also known to stimulate RNA polymerase I-dependent promoters and upregulate rRNA synthesis and ribosome biogenesis [49]. However, the molecular mechanisms underlying the activation of rDNA transcription by HBx remain to be elucidated. Since, HCC patients with HBV infection show a strong positive correlation between elevated NPM levels and poor prognosis [61], NPM could serve as a potential link between increased production of ribosomes and progression of HBV-mediated HCC.

Abbreviations: BrdU, 5-Bromo-2′-deoxy-uridine; ChIP, chromatin immunoprecipitation; HBV, hepatitis B virus; HBx, X protein of HBV; HCC, hepatocellular carcinoma; IHH, immortalized human hepatocyte; IP, immunoprecipitation; MBP, maltose binding protein; MBP-HBx, MBP–HBx fusion protein; NPM, nucleophosmin; PCR, polymerase chain reaction; rDNA, ribosomal DNA; RPs, ribosomal proteins; RT-qPCR, real-time quantitative PCR; WB, western blotting; XE, HBx-specific shRNA

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Here, we investigated the oncogenic cooperation between viral HBx and cellular NPM. We report a direct interaction between HBx and NPM which was associated with the stabilization of NPM and nucleolar accumulation of the viral oncoprotein. The nucleolar HBx facilitated nucleosome disassembly at the rDNA promoter and stimulated rRNA synthesis in an NPM-dependent manner. Thus, the present study shows how a viral oncoprotein can subjugate NPM functions in order to control ribosome biogenesis and induce cancer phenotype.

2. Materials and methods

2.1. Expression plasmids, shRNA and reporter constructs

Eukaryotic expression vectors for wild type HBx and its deletion mutant, X9 have been described previously [30]. FLAG-tagged WT-NPM and its mutants were kindly provided by Prof. Charles Sherr [3,31]. His-NPM prokaryotic construct was developed by cloning WT–NPM sequence at *EcoR*I–*Xho*I restriction sites in pET28a. HBx-specific shRNA (XE) [22], MBP–HBx [50] and luciferase reporter construct rDNA-pGL3 [49] have been previously reported.

2.2. Cell culture and DNA transfection

Human hepatoma Huh7 cells (kindly provided by Dr. Aleem Siddiqui, University of Colorado, Denver), Immortalized Human Hepatocytes (IHH; kindly provided by F. Danniel), Human liver carcinoma HepG2 (ATCC HB-8065) and its derivative HepG2.2.15 cells (a kind gift from Dr. S. Kamili, CDC, Atlanta) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 10% CO_2. Cells in 60-mm (0.6×10^6 cells) and 100-mm (0.8×10^6 cells) culture dishes were transfected respectively with 2 and 5 μg of indicated plasmids using Lipofectamine (Invitrogen, USA) according to manufacturer's protocol. For luciferase reporter assays, 0.5 μg of luciferase reporter construct was co-transfected with 1.5 μg of the indicated expression plasmids in 6-well culture plates (0.4×10^6 cells/well).

2.3. Chemical reagents and antibodies

The chemical inhibitors MG132 (40 μM for 6 h), cycloheximide (100 μg/ml for 15 min) and CDK2 inhibitor II (10 μM for 6 h) were obtained from Calbiochem (EMD Biosciences, USA). Thymidine (Sigma-Aldrich, St. Louis, MO) was used at a concentration of 2 mM for 10–12 h. Etoposide (Sigma-Aldrich) was used at a concentration of 50 μM for 24–48 h. Antibodies against NPM (B23), UBF, HBx, ubiquitin, c-Myc, HA-epitope, Rb, pRb (Ser^{807}) and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against pNPM (Thr^{199}) and RNA polymerase I were procured from Abcam (Cambridge, MA, USA). Mouse antibody against FLAG-tag was obtained from Sigma. ChIP grade antibodies against total and acetylated histones were procured from EMD Millipore (Billerica, Massachusetts, USA). Other chemical reagents used were 4′,6-diamidino-2-phenylindole (DAPI, Molecular probes, Invitrogen, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Heparin (Sigma-Aldrich), Luciferase assay system (Promega, Madison, WI), Moloney Murine Leukemia Virus Reverse Transcriptase (Fermentas, Waltham, MA, USA), SYBR Green Mix (Bio-Rad, USA). All the restriction enzymes used were from Fermentas.

2.4. Immunoprecipitation and western blotting

Western Blotting (WB) and immunoprecipitation (IP) for protein samples were done as described previously [41].

2.5. RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) following manufacturer's instructions. cDNA was prepared using either random hexamer (for rRNA) or oligo-dT primer (for mRNA) as described earlier [49]. RT-qPCR was performed using SYBR Green mix on StepOnePlus platform (Applied Biosystems, Foster City, California, USA) as described previously [23] with specific primers (Supplemental Table S1). The results were analyzed using the comparative C_T method [47].

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [52]. The eluted chromatin was analyzed either by semi-quantitative PCR or SYBR green based real-time qPCR with the indicated primer sets (Supplemental Table S1). For all ChIP assays, cells were serum-starved for 60 h and harvested after 9 h of serum stimulation.

2.7. Luciferase assay

Luciferase assay was performed using luciferase assay system (Promega) as per manufacturer's instructions. Relative luciferase activity was calculated after normalizing with total protein in the samples. The results are expressed as mean ± SD of three independent experiments.

2.8. Cell synchronization and fluorescence-activated cell sorting (FACS)

Huh7 cells were synchronized by double thymidine block (2 mM) after 24 h of transfection as reported previously [46]. Treated cells were harvested, fixed and stained with propidium iodide (50 μg/ml) [29] and flow cytometry was performed using FACSCalibur (Becton, Dickinson and Company, New Jersey, USA). Cell cycle analysis was done using FlowJo software version 4.5.4.

2.9. X15-myc transgenic mouse model

The development of X15-*myc* transgenic mouse model of HCC has been reported earlier [32]. Liver tissue samples of control and transgenic mice of indicated age groups were collected and processed for western blotting [23].

2.10. In vitro protein–protein interaction study

Recombinant MBP and MBP–HBx fusion proteins were expressed and purified as described previously [50]. The recombinant His-NPM protein was expressed using pET-28a vector and was affinity purified with Ni–NTA resin. Purified His-NPM protein was dialyzed against binding buffer (1× PBS, 0.4% Triton X-100 and protease inhibitor cocktail). For each assay, 50 μg of purified His-NPM was incubated with 50 μl bed volume of Ni–NTA Superflow resin (Qiagen, Limburg, Netherlands) in binding buffer at 4 °C for 2 h after which the beads were washed thrice with binding buffer. Beads were then incubated with either 10 μg MBP or MBP–HBx in 2 ml binding buffer for 16 h at 4 °C. Finally, beads were washed 5 times with 1 ml binding buffer and the bound proteins were eluted by boiling the beads in 2× Laemmli buffer. The samples were resolved by SDS-PAGE followed either by staining with Coomassie brilliant blue or by immunoblotting with α-HBx or α-NPM antibodies.

2.11. Immunofluorescence assay

Huh7 cells were seeded on coverslips in a 12-well plate (0.1×10^6 cells/well) and transfected with indicated expression plasmids. After 48 h, cells were processed for immunofluorescence assay as described previously [13]. Fluorescent Alexa Fluor 488 and 594 conjugated secondary antibodies (Molecular probes, USA) were

used. The coverslips were mounted on glass slides with DAPI anti-fade solution to visualize the nucleus. The images were captured with a ×60 objective with NIKON A1R confocal microscope (Nikon, Japan).

2.12. Cell viability assay

IHH cells seeded in 6-well plates were transfected with indicated plasmids. 24 h post-transfection, cells were treated with 50 μM etoposide for 48 h following which the cells were processed for MTT assay to determine the viability of cells as described earlier [6].

2.13. Ribosome profiling

Ribosome profiling was carried out as described previously [17] with some modifications. Briefly, Huh7 cells were transfected with indicated expression plasmids and were incubated with 100 μg/ml cycloheximide for 15 min prior to harvesting. The cells were lysed by resuspension in polysome extraction buffer (20 mm Tris–HCl pH 7.5, 50 mm KCl, 10 mm $MgCl_2$, 1 mM DTT, 100 μg/ml Heparin, 1% Triton X-100 and protease inhibitor cocktail) followed by vortexing. After incubation on ice for 1 h, the lysates were centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatants were collected. The samples were then fractionated on sucrose gradients of 10–50% consistency followed by centrifugation at 38,000 rpm for 2 h at 4 °C. Fractions were collected followed by spectrophotometry at 254 nm.

2.14. Colony formation assay

IHH cells transfected with indicated expression vectors were trypsinized 48 h post-transfection and soft agar colony formation assay was performed as described earlier [26]. Bright field images of colonies formed were captured with a ×10 objective with Nikon Eclipse T*i* inverted microscope (Nikon, Japan). The number of colonies formed were counted and their mean (n = 3) was determined.

2.15. Statistical analysis

Statistical significance of the results was tested by one-way ANOVA (Analysis of variance) followed by post-hoc comparisons using Tukey's HSD (honestly significant difference) test using Sigma plot 12.0 (www.sigmaplot.com). Quantitative data obtained from densitometric analysis of western blots and Coomassie stained SDS-PAGE gels are shown as mean ± standard deviation of three independent experiments along with their *p*-values in supplemental Table S2. Data were considered significant at $p < 0.05$ (*), $p < 0.01$ (**) and highly significant at $p < 0.001$ (***).

3. Results

3.1. HBx modulates NPM expression during the S phase of cell cycle

As NPM level is known to be upregulated in many human cancers including HCC, we wondered if a viral oncoprotein like HBx could induce NPM expression in the hepatic microenvironment. The level of NPM was measured after transfecting human hepatoma Huh7 cells and immortalized human hepatocytes (IHH) cells with the HBx expression vector. NPM protein levels were found to be upregulated in the presence of HBx (Fig. 1A). Concurrently, a marked increase in the NPM protein level was also observed in HepG2.2.15 cells (Fig. 1B) that carry the HBV genome integrated into the human hepatoma HepG2 cells and express HBx protein [48]. Consistent with these observations, nearly 3–5 fold increase in the NPM protein levels was also observed in the liver tissues of three and nine months old X15-*myc* transgenic mouse model of HCC (Fig. 1C). No appreciable change in the levels of NPM transcript was seen under these conditions (Supplemental Fig. S1).

As NPM is majorly required during the replicative (S) and mitotic (M) phases of the cell cycle [37,44], we next analyzed the effect of HBx on NPM expression during different phases of the cell cycle. Huh7 cells synchronized at the G1/S transition using double thymidine block

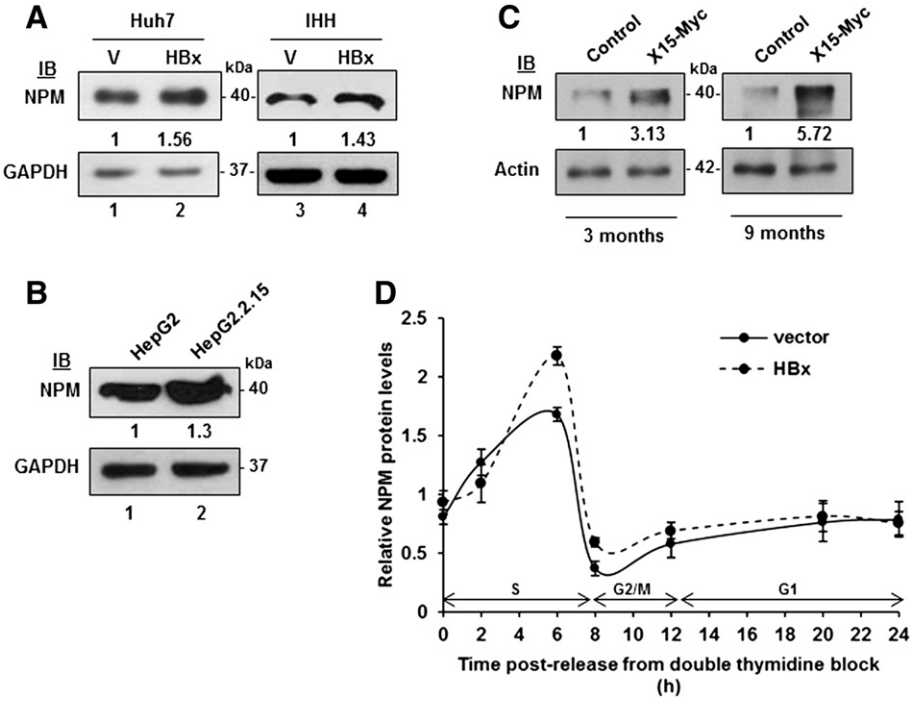


Fig. 1. Effect of HBx on NPM expression. (A) Huh7 and IHH cells were transfected with the vector control (V) or HBx expression plasmid and the levels of NPM protein were measured by western blotting. (B) NPM protein levels measured as above in HepG2 and HepG2.2.15 cells. GAPDH was used as an internal control. (C) Liver tissue lysates from the control and *X15-myc* transgenic mice model of different age groups (3 months and 9 months) were immunoblotted for NPM. Actin was used as a loading control. The blots shown are representative of three independent experiments. Relative expression levels of NPM protein are shown below the corresponding panels. (D) Huh7 cells transfected with either vector control or HBx expression plasmid were synchronized by double thymidine block. The cell lysates were collected at the indicated time points after release and immunoblotted for NPM and GAPDH. Graph shows the mean relative levels of NPM obtained from three independent experiments after normalization with GAPDH. Error bars represent ± standard deviation (S.D.); n = 3.

showed a marked increase in NPM expression in the presence of HBx particularly during the S-phase, whereas the expression was maintained at a basal level during other phases of cell cycle (Fig. 1D, Supplemental Fig. S2A and S2B).

3.2. HBx stabilizes NPM by enhancing its phosphorylation

Since HBx is reported to potentiate the activity of CDK2 kinase [41] which phosphorylates NPM at Thr^{199}[55], we next monitored the phosphorylation status of NPM in its presence. We found a marked increase in the phosphorylation of NPM at Thr^{199} in the presence of wild-type HBx in contrast to its mitogenic signaling domain mutant X9 [41], which failed to induce the phosphorylation (Fig. 2A and Supplemental Fig. S3). Since, the total NPM protein level also increased upon HBx expression, we wondered if CDK2-mediated NPM phosphorylation at Thr^{199} has any role in promoting the intracellular stability of NPM. The stability of NPM is well known to be regulated *via* its cleavage by caspase-3 [7]. Therefore, we monitored the proteolytic cleavage of NPM under these conditions. We found a strong negative correlation between Thr^{199} phosphorylation of NPM and its cleavage as increase in phosphorylation of NPM at Thr^{199} in the presence of HBx decreased the amount of 21 kDa cleaved form of NPM (Fig. 2A). On the other hand, mutant X9 that does not potentiate CDK2 activity, failed to confer intracellular stability to NPM (Fig. 2A and Supplemental Fig. S3).

To establish the role of CDK2 in NPM stabilization, HepG2 and HepG2.2.15 cells were treated with CDK2 inhibitor and the levels of Thr^{199} phosphorylated, full length and proteolytically cleaved forms of NPM were measured. Treatment with CDK2 inhibitor led to a significant decline in the levels of full length NPM with a concomitant increase in

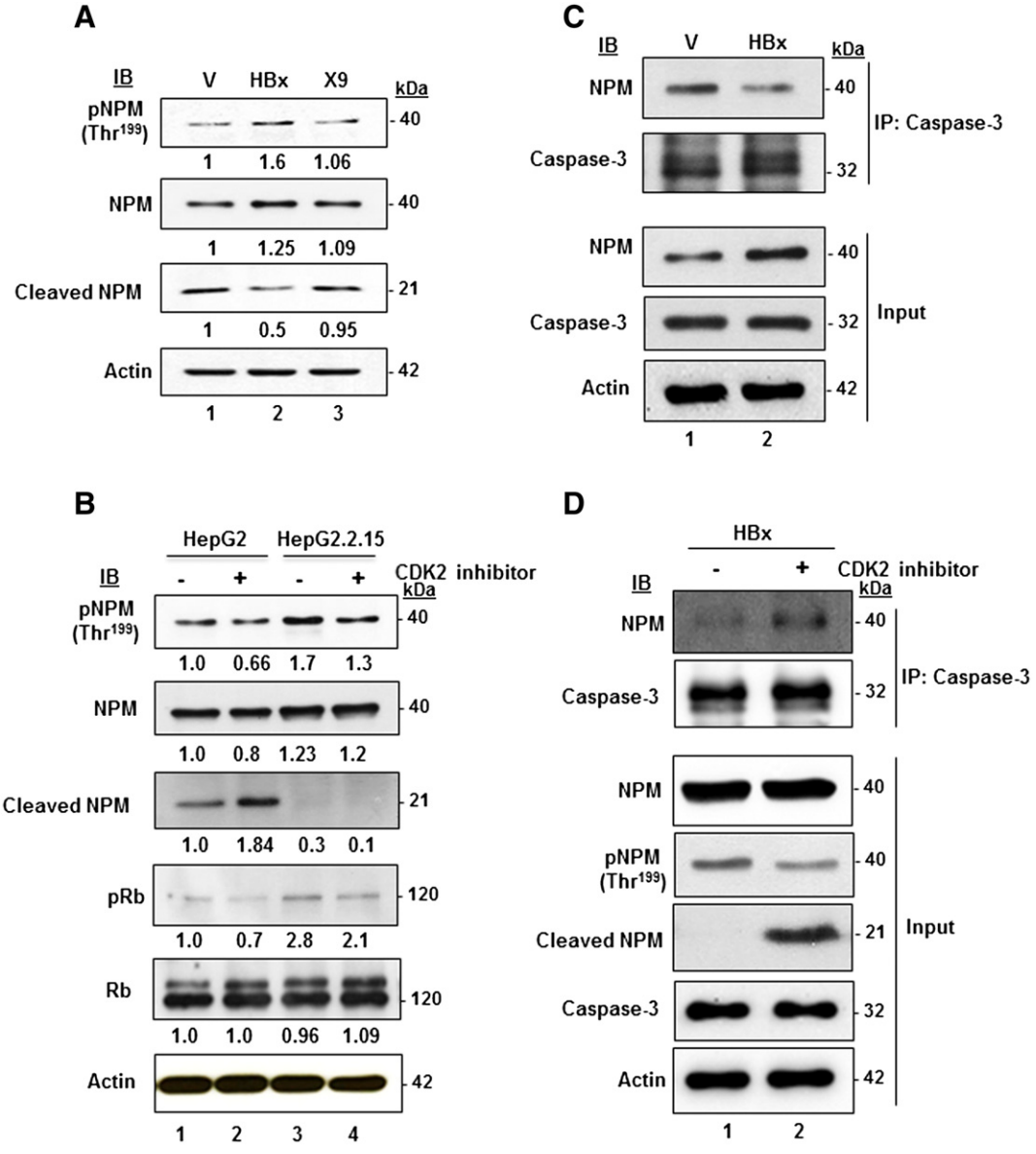


Fig. 2. Phosphorylation and stability of NPM in the presence of HBx. (A) IHH cells were transfected with either vector control (V), wild-type HBx expression vector (HBx) or its mutant X9 and the levels of total, phosphorylated and cleaved NPM were measured by western blotting. (B) HepG2 and HepG2.2.15 cells were treated with CDK2 inhibitor II (10 μM, Calbiochem) and the cells were harvested 6 h post-treatment. Protein levels of total, cleaved and Thr^{199} phosphorylated NPM were analyzed by SDS-PAGE and western blotting. Relative expression levels of total, cleaved and phosphorylated NPM under different conditions are shown below the corresponding panels. Phosphorylated Rb was used as a control for the inhibition of CDK2 activity. (C) Interaction of caspase-3 and NPM. Lysates of IHH cells transfected with either vector control or HBx expression plasmid were immunoprecipitated with anti-caspase-3 antibody and immunoblotted for NPM and caspase-3. (D) Interaction of caspase-3 and NPM in the presence or absence of CDK2 inhibitor in HBx-transfected IHH cells. The cell lysates were immunoprecipitated with anti-caspase-3 antibody and immunoblotted for caspase-3, phospho-, cleaved- and total NPM. Actin was used as an internal control in all the experiments. The blots shown are representative of three independent experiments.

its proteolytically cleaved fragment in both HepG2 and HepG2.2.15 cell lines (Fig. 2B).

To further understand the molecular mechanisms underlying HBx-mediated intracellular stabilization of NPM, we checked the interaction of NPM with caspase-3 in the presence of HBx. Interestingly, we found a marked reduction in their interaction in HBx-expressing cells as compared to the vector control (Fig. 2C). Further, we checked if NPM phosphorylation at Thr^{199} altered the interaction between caspase-3 and NPM. Intriguingly, inhibition of CDK2-mediated NPM phosphorylation enhanced the interaction between the two proteins (Fig. 2D) resulting in the elevated levels of proteolytically cleaved NPM.

3.3. HBx exerts its anti-apoptotic effect by preventing the proteolytic degradation of NPM

NPM overexpression is known to be associated with abrogation of apoptosis [10,59]. The C-terminal region of NPM is considered indispensable for eliciting its anti-apoptotic effects [33] as its cleavage by activated caspase-3 leads to abrogation of its anti-apoptotic function [7]. Since viral HBx is well known to confer resistance to genotoxic agents and interfere with apoptosis in cancer cells [24,42], we wondered if NPM was one of the mediators of anti-apoptotic activity of HBx. We used two apoptosis inducers in our experiments, *viz*., etoposide which is a well-known DNA intercalating agent and a topoisomerase II inhibitor, and paclitaxel which is a common mitotic inhibitor. Treatment of cells with etoposide enhanced the proteolytic degradation of NPM as observed by the increase in levels of cleaved NPM fragment (Fig. 3A,C). However, there was a marked inhibition in the proteolytic cleavage of NPM upon HBx expression (Fig. 3A,C). Similarly, HBx also conferred resistance to proteolytic cleavage of NPM in paclitaxel-treated cells (Fig. 3B). Further, increase in the viability of HBx-transfected cells even in the presence of an apoptotic inducer (as observed by MTT assay) confirmed that etoposide-induced apoptosis could be abated in the presence of HBx (Fig. 3D). These results strongly suggested that NPM plays a crucial role in mediating the anti-apoptotic effects of viral HBx.

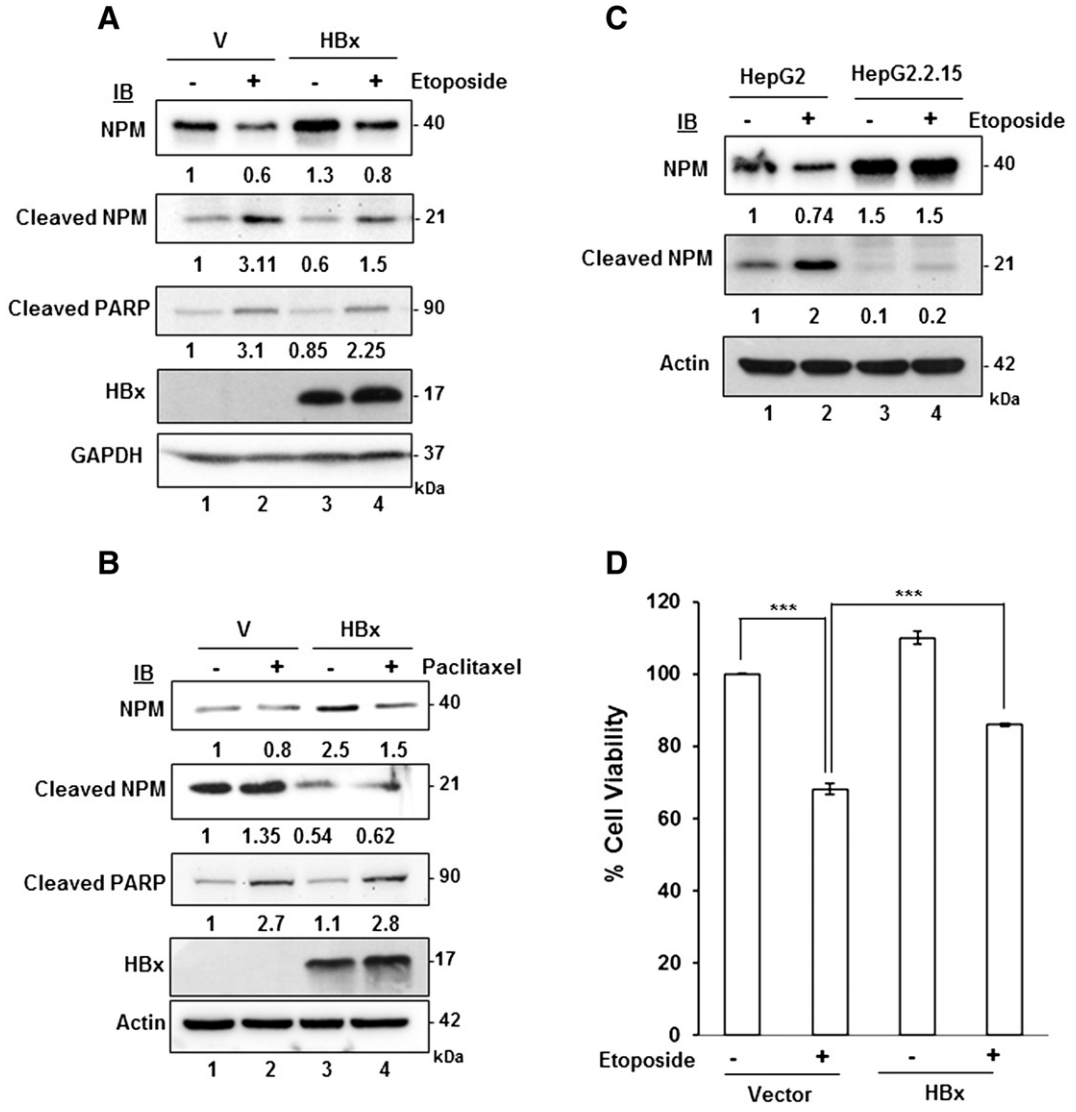


Fig. 3. NPM as a mediator of the anti-apoptotic effects of HBx. IHH cells transfected with either vector control (V) or HBx expression plasmid were treated with 50 μM Etoposide (A) or 6 nM Paclitaxel (B) for 24 h. The protein levels of full length as well as cleaved form of NPM were then analyzed by western blotting. Cleavage of PARP was measured as a positive control for etoposide and paclitaxel treatment. GAPDH and actin were used as loading controls, respectively. (C) HepG2 and HepG2.2.15 cells were treated with etoposide for 24 h as above and the cell lysates were immunoblotted for full length and cleaved form of NPM. Actin was used as an internal control. All the blots shown are representative of three independent experiments. Relative expression levels of different proteins are shown below each panel. (D) Vector and HBx-transfected IHH cells were treated with etoposide 24 h after transfection and the viability of cells was determined by MTT assay. Percent survival of cells was measured and compared to the vector-transfected untreated cells (taken as 100%). The data are shown as mean ± S.D. of three independent experiments. ***, statistically significant difference ($p < 0.001$) as obtained *via* one-way ANOVA followed by Tukey's HSD test.

3.4. HBx directly interacts with NPM and gets translocated into the nucleolus

NPM behaves as a nuclear chaperone and translocates several regulatory proteins such as c-Myc, RPS9 and ARF into the nucleolar compartment [27,35,37]. It also plays an essential role in the nucleolar translocation of several viral proteins, thereby making them competent to alter nucleolar functions upon viral infections [1,53,57,58]. Therefore, to check whether HBx is associated with NPM in the cellular milieu, the lysates of vector or HBx-transfected cells were immunoprecipitated with anti-NPM antibody followed by immunoblotting for HBx. As shown in Fig. 4A, NPM seemed to specifically interact with HBx and the domain of interaction was mapped to the C-terminal region of NPM as none of its C-terminal deletion mutants (NPM C1, NPM C2 and NPM C3) could immunoprecipitate HBx (Fig. 4B and C). Interestingly, the NPMc mutant, with an extra nuclear export signal, also interacted with HBx with even higher affinity (Fig. 4B, lane3). The increased interaction of NPMc mutant with HBx observed in the present study might be a result of its unfolded tertiary structure at the C-terminus which has also been shown to enhance its interaction with CRM1 nuclear export receptor [21]. Together, these results suggested that the C-terminal nucleic acid-binding domain of NPM is involved in its interaction with HBx.

To validate the possibility that the interaction between viral HBx and cellular NPM was through a direct physical interaction and that it did not involve other factors, we used purified recombinant His-tagged NPM protein to pull down the purified recombinant MBP–HBx fusion protein. As shown in Fig. 4D, the purified His-NPM protein was able to pull down MBP–HBx fusion protein but failed to pull down recombinant MBP. Thus, NPM interacted directly with HBx.

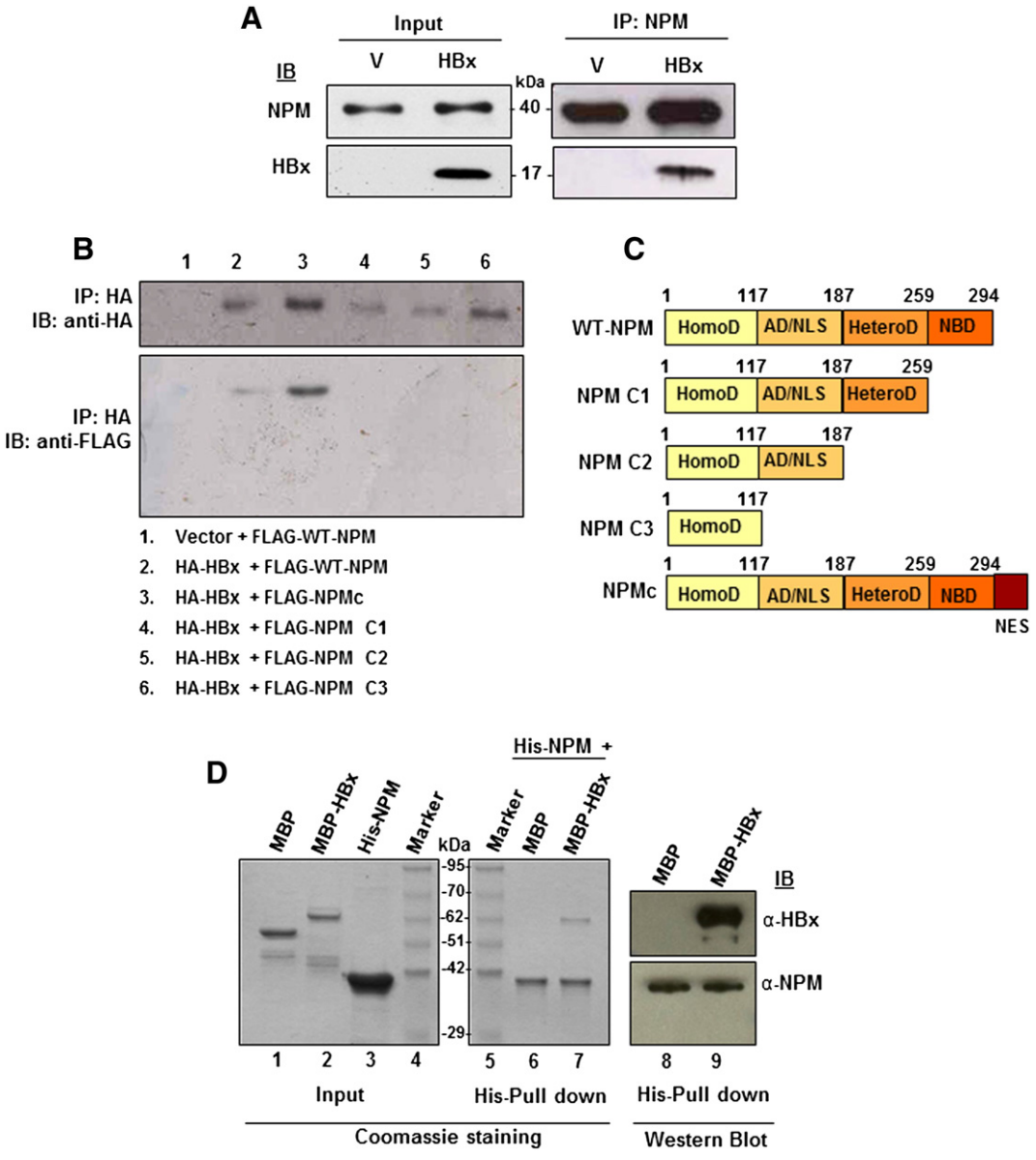
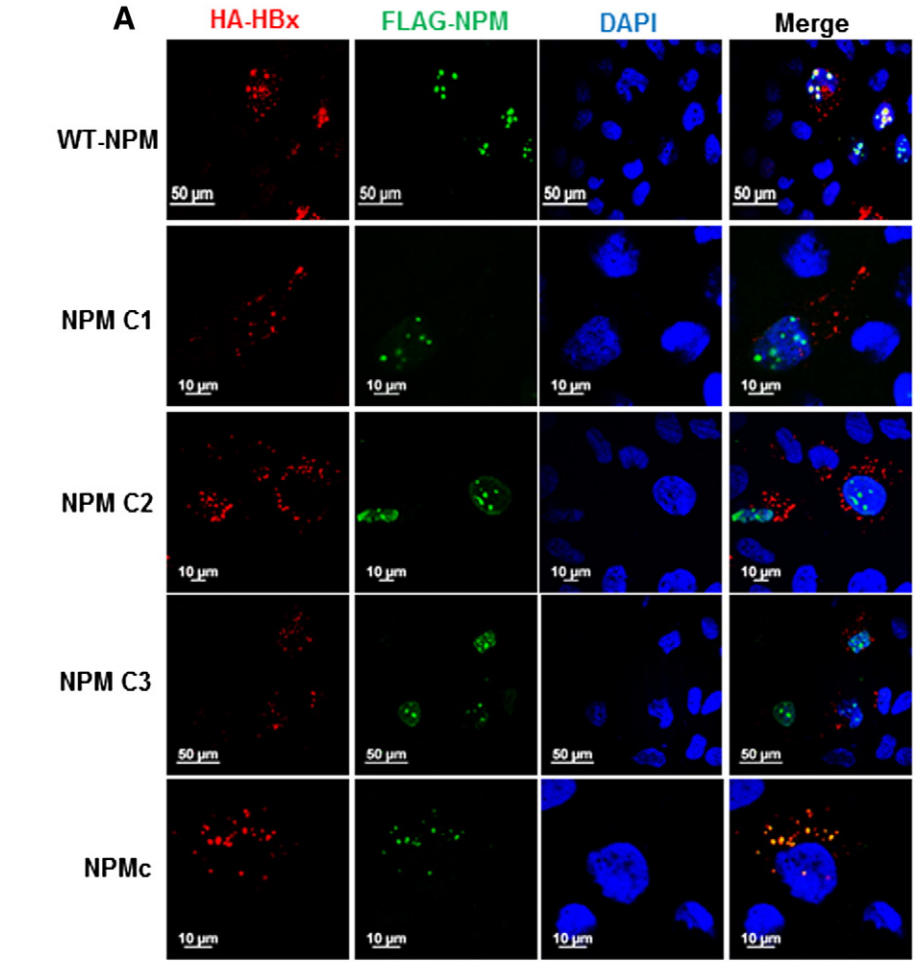


Fig. 4. Direct interaction between NPM and viral HBx. (A) Huh7 cells transfected with HBx and vector control were subjected to immunoprecipitation (IP) using anti-NPM antibody, followed by immuno-blotting (IB) for HBx. (B) To map the NPM domain(s) of interaction, cells were co-transfected with the indicated FLAG-tagged NPM mutants and HA-tagged HBx, and were subjected to IP using anti-HA antibody followed by IB analysis using anti-FLAG antibody. (C) Schematic representation of the domain structures of WT–NPM and its deletion mutants. HomoD: homo-dimerization domain; AD/NLS: acidic domain/nuclear localization signal; HeteroD: hetero-dimerization domain; NBD: nucleic-acid binding domain. (D) *In vitro* binding assay of recombinant MBP–HBx and His–NPM. The His-NPM protein was allowed to bind to the Ni–NTA beads and incubated with either MBP (lane 6) or MBP–HBx (lane 7). The beads were boiled in 2× Laemmli buffer and the eluted proteins were resolved *via* SDS-PAGE followed by staining with Coomassie brilliant blue (lanes 6,7) or immunoblotting with NPM and HBx antibodies (lanes 8,9). Lanes 1–3 represent purified recombinant MBP, MBP–HBx and His-NPM proteins respectively. Results shown are representative of three independent experiments.



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NPM	NPM localization	HBx localization	Interaction with HBx
WT-NPM (overexpressed / endogenous)	Nucleoli	Cytoplasm, nuclear, nucleolar	+
NPM C1	Nucleoli/ Nucleoplasm	Cytoplasm	-
NPM C2	Nucleoli/ Nucleoplasm	Cytoplasm	-
NPM C3	Nucleoli/ Nucleoplasm	Cytoplasm	-
NPMc	Cytoplasm	Cytoplasm	+

Fig. 5. Sub-cellular localization of viral HBx upon NPM over-expression. (A) Huh7 cells co-transfected either with FLAG tagged wild-type NPM (WT-NPM) or its mutants along with HA tagged-HBx were examined by confocal immunofluorescence microscopy after staining with antibodies to FLAG and HA. Fluorescent Alexa Fluor 488 and 594 conjugated secondary antibodies were used for detection of FLAG-tagged NPM (green) and HA–HBx (red), respectively. Nucleus stained with DAPI has been shown in blue. (B) Immunofluorescence assay of HepG2.2.15 cells after staining for endogenous NPM (green) and viral HBx (red) as above. The images shown are representative of three independent experiments. (C) Summary table showing the localization of HBx in the presence of WT–NPM and its C-terminal deletion mutants.

We next investigated the consequence of HBx–NPM interaction on the sub-cellular distribution of HBx using immunofluorescence assay. HBx was found to localize mainly in the cytoplasmic compartment. However, a fraction of it was also present in the nuclear as well as nucleolar regions (Supplemental Fig. S4). Overexpression of wild-type NPM facilitated the translocation of HBx into the nucleolar compartment (Fig. 5A, top panel). Expectedly, co-expression of HBx with the C-terminal deletion mutants of NPM that did not interact with HBx, failed to translocate cytoplasmic HBx into the nucleolus (Fig. 5A). Interestingly, HBx co-localized in the cytoplasmic compartment with the NPMc mutant (Fig. 5A, bottom panel), which has an extra NES motif and localizes in the cytoplasm [31]. Similarly, the HBx-expressing HepG2.2.15 cells also showed co-localization of HBx and endogenous NPM in the nucleolus (Fig. 5B). Together these results suggested a novel role of NPM in the nucleolar translocation of HBx. Fig. 5C summarizes the localization of HBx with respect to NPM and its deletion mutants.

3.5. HBx–NPM interaction enhances rDNA transcription in the nucleolus

The physiological relevance of HBx–NPM interaction and the associated nucleolar localization of HBx was studied vis-à-vis ribosomal DNA transcription. Co-expression of HBx and wild-type NPM led to a significant increase ($p < 0.001$) in the RNA polymerase I-dependent luciferase reporter activity (Fig. 6A). The reporter assay results were substantiated by a significant elevation in the levels of rRNA transcripts ($p < 0.001$) under these conditions (Fig. 6B). In contrast, co-expression of HBx with the NPM deletion mutants did not stimulate the rDNA promoter (Fig. 6A,B). Note that the apparent increase in luciferase activity in cells co-expressing NPM C1 mutant and HBx, over HBx-transfected cells was found to be statistically non-significant ($p < 0.117$). Similar results were obtained when rRNA levels were normalized using actin as an internal control (Supplemental Fig. S5). Thus, HBx and NPM seem to cooperate in stimulating rDNA promoter which may have a role in the pathophysiology of HBV-mediated HCC.

3.6. HBx facilitates the recruitment of RNA polymerase I transcription machinery on the rDNA promoter in an NPM-dependent manner

To further substantiate the enhancement of rDNA transcription in the presence of HBx, we analyzed the recruitment of different transcription factors to their cognate response elements on the rDNA promoter. As revealed by chromatin immunoprecipitation (ChIP) assays, a significant two-fold increase in the enrichment of c-Myc and UBF was observed in the presence of HBx as compared to the vector control (Fig. 7A,B). The binding of c-Myc and UBF was enhanced by many folds upon co-expression of wild-type NPM and HBx. However, no change in the promoter occupancy of these transcription factors was observed in the presence of NPM C3 mutant and HBx with respect to the vector control. Interestingly, it was found to be even lower as compared to cells expressing HBx alone, effectively reversing the effects elicited by HBx. Since NPM is functional as an oligomer (often as a pentamer), the expression of the dominant negative NPM C3 mutant [3,34] can result in its oligomerization with the endogenous NPM leading to the formation of non-functional NPM C3-NPM oligomers. Therefore, the simultaneous expression of HBx with NPM C3 mutant might prevent the utilization of endogenous NPM by HBx thereby precluding HBx from exerting its effects on the recruitment of c-Myc and UBF onto the rDNA promoter (Fig. 7). Thus, these results supported our earlier observations on the role of NPM in HBx-mediated activation of rDNA transcription.

UBF along with other transcription factors such as c-Myc, is well known to promote the recruitment of RNA polymerase I and thus determine the relative proportion of active and silent rRNA genes [11]. Therefore, we next checked the effect of HBx expression on the occupancy of RNA polymerase I on the rDNA promoter. Interestingly, the RNA Pol I occupancy was enhanced by nearly fourfolds in HBx transfected Huh7 cells as compared to the vector control (Fig. 7C). Expectedly, co-expression of wild-type NPM and HBx showed a further increase in Pol I binding whereas no increase in its promoter occupancy was observed when dominant negative NPM C3 mutant was co-expressed with HBx (Fig. 7C). Interestingly, the knockdown of HBx expression by specific shRNA construct (XE), led to a pronounced decrease in the rDNA promoter occupancy of transcription factors such as c-Myc along with RNA Pol I (Fig. 7D). Together, these results suggested that NPM is an important cellular partner of viral HBx in stimulating rDNA promoter activity and hence Pol I transcription.

3.7. HBx promotes rDNA transcription by enhancing NPM acetylation and affecting nucleosome occupancy

We next sought to further delineate the molecular mechanism of enhanced recruitment of Pol I transcription machinery on the rDNA promoter in the presence of HBx. Previously, it has been found that

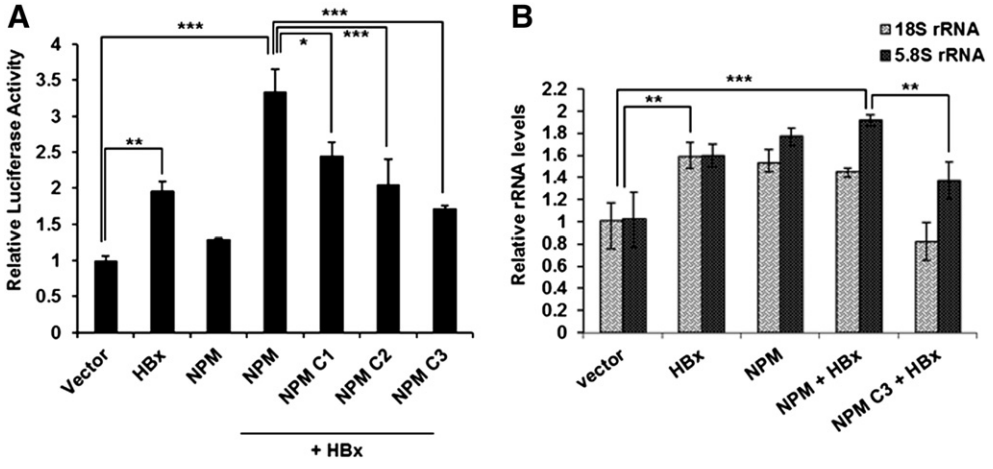


Fig. 6. NPM-dependent stimulation of rDNA promoter in the presence of HBx. (A) Huh7 cells were transfected with rDNA-pGL3 luciferase reporter construct along with the expression vectors for HBx, FLAG-NPM and its C-terminal deletion mutants NPM C1, NPM C2 and NPM C3 as indicated. Cells were harvested 48 h after transfection and the cell extracts were assayed for luciferase activity. The graph represents the relative luciferase activity after normalization with the total protein. (B) Cells were transfected with either HBx alone or in combination with FLAG-NPM or its C-terminal deletion mutant NPM C3 as indicated. Total RNA was extracted after 48 h and was used to measure the levels of 5.8S and 18S rRNA by RT-qPCR using specific primers (Supplemental Table S1). The transcripts for acidic ribosomal phosphorprotein P0 were used as an internal control. The data are shown as mean ± S.D. of three independent experiments. *, **, ***, represents statistically significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively as obtained *via* one-way ANOVA followed by Tukey's HSD test.

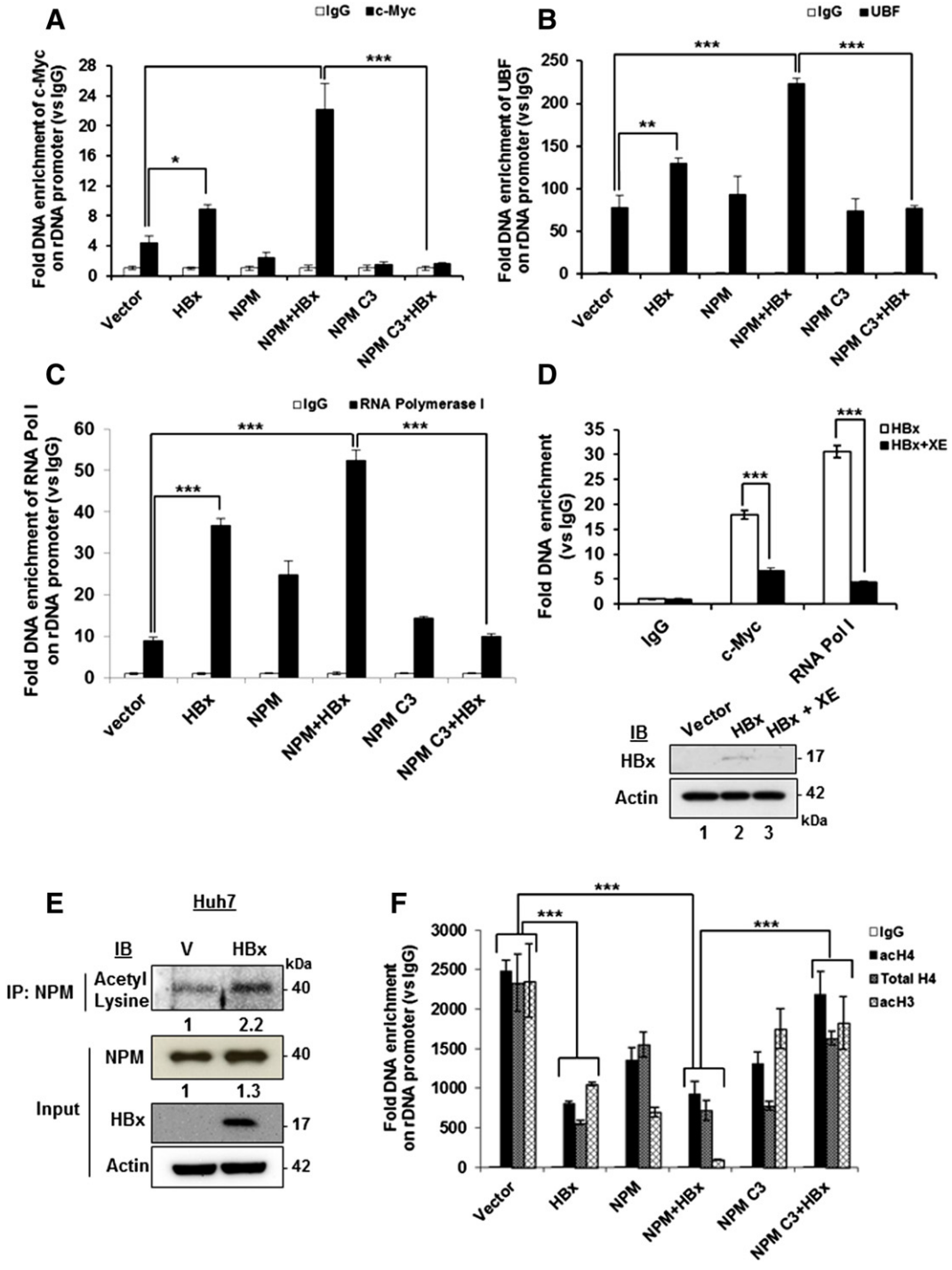


Fig. 7. Effect of HBx on the recruitment of RNA polymerase I transcription machinery on the rDNA promoter. Huh7 cells transfected with vector control or expression plasmids for HBx, WT–NPM, and NPM C3 mutant either alone or in combination as indicated were grown in serum-free medium for 60 h. The cells were released by addition of complete medium and harvested after 9 h for analyzing the promoter occupancy of the following factors: c-Myc (A), Upstream Binding Factor or UBF (B) and RNA polymerase I (C). (D) Cells were transfected with vector control or HBx expression plasmid either alone or in combination with HBx-specific shRNA (XE) and analyzed for the rDNA promoter occupancy of c-Myc and RNA Pol I by ChIP-qPCR as above. The lower panel shows the expression of HBx under these conditions. (E) Cells transfected with either vector control or HBx expression plasmid were harvested after 48 h of transfection and the cell lysates were immunoprecipitated with anti-NPM antibody followed by western blotting for acetylated NPM using anti-acetyl Lysine antibody. Actin was used as an internal control. The fold change in levels of acetylated NPM (acNPM) and NPM are shown below the corresponding panels. The blots shown are representative of three independent experiments. (F) Huh7 cells were transfected with vector control or expression plasmids for HBx, WT–NPM, and NPM C3 mutant either alone or in combination as indicated and were analyzed for the relative promoter occupancy of total histone H4, acetylated histone H4 (acH4) and acetylated histone H3 (acH3) over that of IgG control by ChIP-qPCR using specific primers (Supplemental Table 1). The data are shown as mean ± S.D. of three independent experiments. *, **, ***, represents statistically significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, as obtained *via* one-way ANOVA followed by Tukey's HSD test.

alteration of histone chaperone activity of NPM, for example, by acetylation is associated with its increased affinity for acetylated histones which in turn triggers nucleosomal disassembly and activation of transcription [51]. Therefore, to understand the mechanism of HBx-mediated enhanced rDNA transcription, we checked the acetylation status of NPM in the presence of HBx. HBx expression resulted in a significant (~2 fold) increase in the levels of acetylated NPM (Fig. 7E). Also, there was a marked decline in the recruitment of acetylated

histone H4 (acH4) on the rDNA promoter in HBx-expressing HepG2.2.15 cells but not in HepG2 cells (Supplemental Fig. S6). Notably, the occupancy of acH4, acH3 and total histone H4 also decreased in cells expressing either HBx or HBx along with NPM (Fig. 7F). Interestingly, the histone occupancy on the rDNA promoter was relatively higher when HBx was co-expressed with NPM C3 mutant (Fig. 7F). Thus, HBx seems to enhance rDNA transcription *via* a novel regulatory mechanism involving the depletion of histones resulting from increased NPM acetylation.

3.8. HBx involves nucleophosmin to promote ribosome biogenesis, cell proliferation and cell transformation

Since rDNA transcription positively correlates with ribosome biogenesis, we next analyzed the ribosome profile of cells upon HBx expression (either alone or in combination with NPM and its mutant NPM C3). A significant increase ($p < 0.01$; $n = 3$) in the amount of mature 80S ribosomes was observed in HBx and NPM co-transfected cells as compared to the control cells (Fig. 8A and B). Further, the amount of ribosomes was drastically reduced when HBx was co-expressed with the NPM C3 mutant. These results signify the importance of NPM in HBx-mediated enhanced ribosome biogenesis.

Further, to investigate whether the observed enhanced ribosome biogenesis had a bearing on cellular proliferation, we analyzed the proliferation rate by measuring the incorporation of bromodeoxyuridine (BrdU) in the cells under these conditions. The percentage of BrdU positive cells was significantly higher in cells transfected with HBx either alone or in combination with wild-type NPM (Fig. 8C). However, there was a marked reduction in the BrdU positive cells when HBx was co-expressed along with the dominant negative NPM C3 mutant. To test whether the enhanced proliferation was also linked to cellular transformation, we performed soft agar colony formation assay to evaluate the growth-promoting role of NPM in the presence of HBx. As shown in Fig. 8D, IHH cells co-transfected with HBx and NPM produced significantly higher number of colonies as compared to the vector control. In contrast, co-expression of HBx with dominant negative NPM C3 mutant did not induce cellular transformation of these cells. Taken together, these results signify the pivotal role played by the host protein NPM in promoting the oncogenic effects of viral oncoprotein HBx.

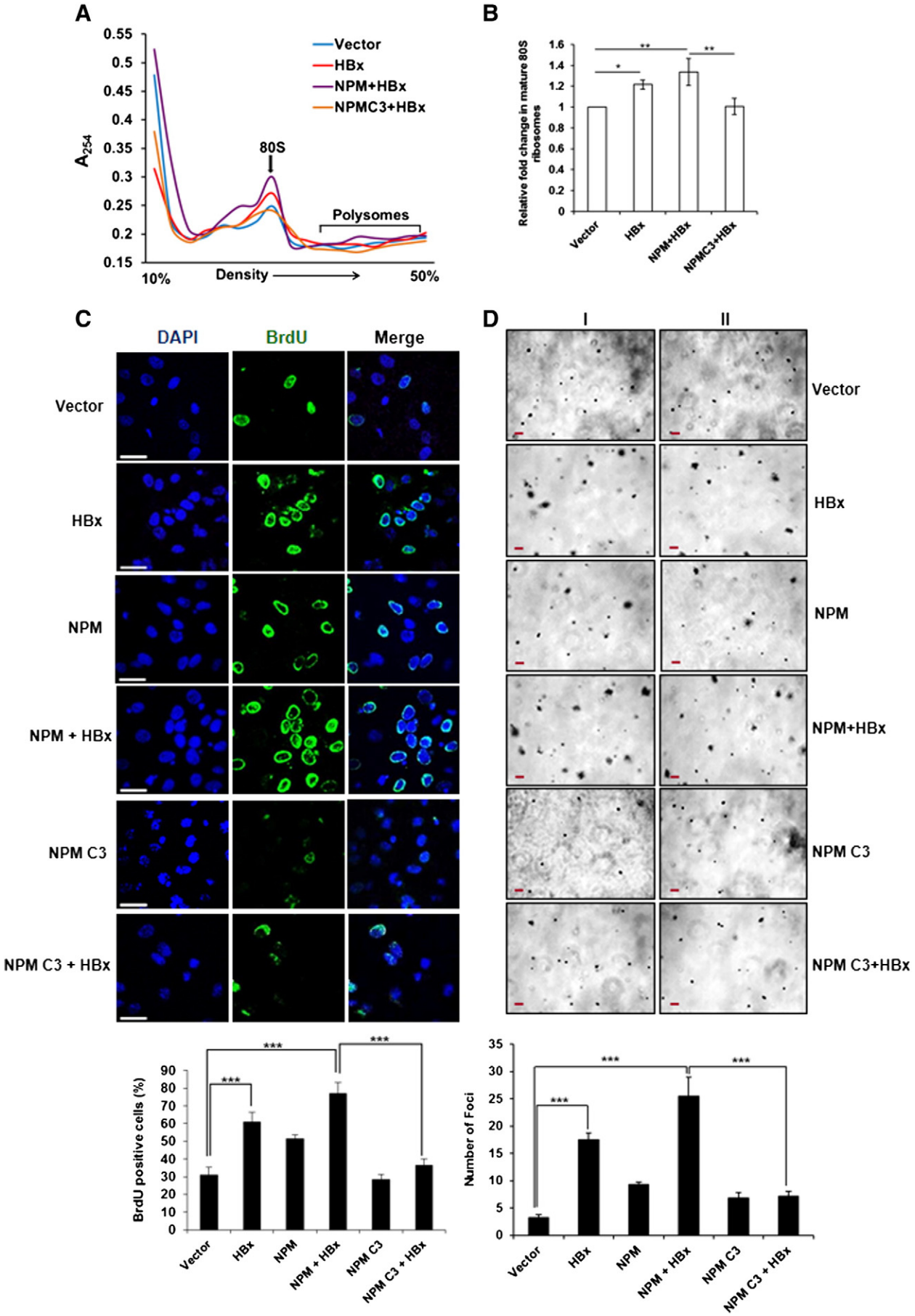
4. Discussion

Nucleolus, earlier considered as an exclusive factory of ribosome biogenesis, has been recently implicated as a sensor of cellular and external stresses due to its involvement in several signaling pathways. Most of these functions are attributed to the major nucleolar proteins *viz*., NPM, nucleolin and fibrillarin which constitute almost 20% of the total nucleolar proteome. Ribosome biogenesis, being the most energy consuming process of the cell, is under an efficient regulation and thus is tightly linked to cellular proliferation. Indeed, rDNA transcription, one of the major events of ribosome biogenesis, is at the center of the complex network linking multiple stress signals with cell growth and proliferation. Several oncogenes are known to potentiate rRNA synthesis resulting in enhanced ribosome biogenesis, which is a pre-requisite of uncontrolled cellular proliferation. Recently, we have shown that the HBx oncoprotein of HBV can cooperate with the cellular oncogene c-Myc to enhance the rDNA promoter activity [49]. Not surprisingly, HBx is widely acknowledged to be involved in the development of HCC. However, the mechanistic link between HBx-mediated cellular transformation and the stimulation of rDNA transcription is rather feeble.

Progression of HCC is associated with an escalated nucleolar functioning and nucleolar hypertrophy, a state characterized by the presence of numerous larger nucleoli [56]. The proliferating cells have been shown to have higher levels of NPM as compared to resting or quiescent cells suggesting the mitogenic potential of NPM [15]. In agreement with these observations, increased NPM expression correlates well with tumor progression in HCC [61]. Further, the proteomic analysis of the HBx transfected human hepatocytes has revealed the upregulation of several nucleolar proteins including NPM [36]. NPM is known to actively contribute towards some specific functions during different phases of the cell cycle including ribosome assembly (in the S-phase) and regulation of centrosome duplication cycle (in the M-phase) [37,44]. Besides, it has previously been shown that an enhanced expression of NPM leads to increased c-Myc mediated rDNA gene transcription while reduction in its endogenous levels fail to stimulate c-Myc dependent rRNA synthesis [35]. In this context, our observation that the expression of NPM is further enhanced during S phase in the presence of HBx (Fig. 1D) gains significance and indicates towards increased ribosome biogenesis under these conditions [49].

Many viral oncoproteins use a common strategy either to stabilize cellular oncoproteins [5] or destabilize tumor suppressor proteins by tinkering with their phosphorylation [19]*via* modulating the activity of various protein kinases and phosphatases involved in cell cycle regulation. For instance, the activity of CDK2 is potentiated in the presence of HBx [41]. In our study, HBx-induced CDK2 activity increased the phosphorylation of NPM at Thr^{199} (Fig. 2) which correlated well with its increased intracellular stability. While NPM phosphorylation at Thr^{199} has been previously reported to regulate centrosome duplication [55], the present study seems to suggest its role in conferring intracellular stability to NPM by preventing its caspase-3 mediated cleavage in HBx-expressing cells. Enhanced phosphorylation of NPM at Thr^{199} by CDK2 kinase in the presence of HBx seemed to play a crucial role in preventing its interaction with caspase-3, leading to the accumulation of intracellular NPM. Furthermore, during apoptosis, the activation of caspase cascade results in the proteolytic cleavage of NPM [7]. Since NPM is essential for maintaining cell viability, its cleavage may result in cell death majorly due to reduction in ribosome biogenesis [8]. Our results have shown that HBx can prevent NPM cleavage even in the presence of apoptotic inducers like etoposide and paclitaxel (Fig. 3). Previously, we have shown that HBx oncoprotein can inhibit apoptosis by activating the Akt signaling-pathway [25]. Further, Akt and other cellular oncoproteins such as Ras have also been shown to inhibit apoptosis by preventing NPM degradation ([7,33]). Therefore, inhibition of proteolytic cleavage of NPM in the HBx-expressing cells could be yet another mechanism of derailing apoptosis and promoting cellular transformation.

Fig. 8. Interference in ribosome biogenesis, cell proliferation and transformation by NPM mutant C3 in HBx-expressing cells. (A) Representative polysomal profiles from Huh7 cells transfected with expression plasmids for vector control, HBx, WT–NPM and dominant negative NPM C3 mutant either alone or in indicated combinations. The absorbance of the fractions collected was measured at 254 nm and plotted. Peaks respresenting 80S ribosomes and polyribosomes (or polysomes) are indicated. The experiment was repeated thrice and a representative image is shown. (B) The relative fold change in the absorbance corresponding to the mature 80S ribosomal fraction was calculated as compared to the vector control and their mean ($n = 3$) was plotted. The data are shown as mean ± S.D. of three independent experiments. *, **, statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively as obtained *via* one-way ANOVA followed by Tukey's HSD test. (C) Huh7 cells transfected with vector control, HBx, WT–NPM, and NPM C3 mutant either alone or in combinations as indicated were synchronized in the early S-phase and labeled with BrdU. Nuclei were stained with DAPI (blue) and FITC-conjugated goat anti-mouse antibody was used for visualizing BrdU incorporation (green). Scale bar represents 50 μm. Percentage of BrdU positive cells in each sample was determined and the mean was plotted. The data (lower panel) are shown as mean ± S.D. of three independent experiments. ***, statistically significant difference at $p < 0.001$ as obtained *via* one-way ANOVA followed by Tukey's HSD test. (D) IHH cells were transfected with expression plasmids as in panel (C) and grown in soft agar for 25 days in a humidified chamber with 10% CO_2 at 37 °C. The colonies obtained were stained using crystal violet and visualized under a bright field microscope. Two random images from each sample are shown. Scale bar represents 20 μm. Fifteen random bright field images of each sample were captured with a ×10 objective and number of foci formed in each were counted and their mean ($n = 3$) was plotted as bar graph (lower panel). ***, statistically significant difference at $p < 0.001$ as obtained *via* one-way ANOVA followed by Tukey's HSD test.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Viral oncoproteins have been shown to interact with several nucleolar proteins and affect their stability and myriad of associated functions including apoptosis, cell cycle regulation, DNA replication and repair, stress response, RNA processing and most importantly ribosome biogenesis [14]. The present study showed that cellular NPM interacted with viral HBx through its C-terminus (Fig. 4) which is required for nucleic acid binding [15] and hence is essential for the chaperoning and RNA processing functions. NPM is primarily localized in the nucleolus due to the presence of a nucleolar localization signal at its C-terminus. Our observation that NPM-HBx interaction led to the nucleolar translocation of HBx suggests the nucleolar chaperoning of HBx by NPM (Fig. 5). Since HBx does not possess a nucleolar localization signal, its presence in the nucleolus suggested some important functional implications. Indeed, we observed a major surge in rDNA transcription upon HBx expression. This effect was specific and was mediated by NPM as the NPM deletion mutants that did not interact with HBx failed to upregulate the transcriptional activity of RNA polymerase I (Fig. 6). Thus, our results, for the first time, implicate NPM in HBx-mediated enhancement of rDNA transcription.

Modulation of chromatin architecture is an important means of transcriptional regulation. NPM, like other histone chaperones, plays an important role in assembly and disassembly of nucleosomes and hence is crucial for the regulation of gene expression. Increase in NPM levels *per se* has no effect on nucleosome occupancy. However, its post-translational modifications, primarily acetylation, alter its affinity towards histones [43,51]. Acetylation of NPM increases its affinity towards acetylated histones leading to the depletion of histones from the chromatin, thereby promoting transcription [51]. Hence, the enhanced acetylation of NPM in HBx-expressing cells and a consequent decrease in the histone occupancy on the rDNA promoter observed in the present study (Fig. 7) provides a mechanistic link into the NPM-dependent and HBx-mediated stimulation of the rDNA promoter. It is well known that a transcriptionally competent chromatin state shows relatively lower nucleosome occupancy in order to facilitate the recruitment of transcription machinery. Not surprisingly, a decreased histone occupancy on the rDNA promoter was associated with increased recruitment of RNA polymerase I transcription machinery onto the rDNA promoter (Fig. 7). Apart from NPM, rDNA transcriptional activation involves the synergistic action of various cellular factors such as c-Myc and UBF among others [13]. Therefore, while NPM acetylation and the associated histone depletion appear to play a major role in HBx-mediated rDNA activation, the simultaneous involvement of other mechanisms cannot be ruled out.

Increased rDNA transcription correlates well with enhanced ribosome biogenesis in actively proliferating and transformed cells [40]. We have shown that NPM plays a major role in driving HBx-mediated cell proliferation as evident from the enhanced production of mature 80S ribosomes and increased rate of BrdU incorporation in cells co-expressing HBx and wild-type NPM (Fig. 8). Also, the ability of these cells to form larger number of colonies in soft agar as compared to cells co-expressing HBx and the non-interacting dominant negative NPM mutant further signifies the role of NPM in mediating the oncogenic potential of HBx (Fig. 8). Thus, NPM appears to be a major player in the HBx-driven HCC progression and tumorigenesis. These results further strengthen the notion that viral oncoproteins often hijack nucleolar functions in order to support the growth and proliferation of transformed cells [20].

4.1. Conclusions

In conclusion, the present study delineates a novel mechanism for HBx-mediated enhanced rDNA transcription and cellular transformation. The functional interaction of viral oncoprotein HBx with a major nucleolar phosphoprotein NPM leading to the stabilization of NPM explains a plausible means of the anti-apoptotic action of HBx during HCC progression. Further, translocation of HBx in the nucleolus together with increased acetylation of NPM in HBx-expressing cells could have important bearing on enhanced rDNA transcription apparently *via* accelerated chromatin remodeling near the rDNA promoter. The indispensable nature of NPM in rRNA synthesis and its targeting by HBx to promote cell survival makes it beneficial for the virus and helps in the maintenance and progression of the cancer phenotype.

Competing interests

The authors declare no competing interests.

Authors' contributions

RA performed the experiments and drafted the manuscript. NRK contributed to the experiments and design of the study. VK conceived the study, designed the experiments and finalized the manuscript.

Transparency document

The Transparency document associated with this article can be found, in the version.

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Appendix A. Supplemental data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.04.012.

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